CHINESE PATENT APPLICATION

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Applicant: Shanghai Institute of Biochemistry, Chinese Academy of Sciences.

Title of Application: Secretary expression of a single chain insulin precursor in yeast and its conversion into human insulin.

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Summary: This invention covers the secretary expression of a single chain insulin precursor in yeast and its conversion to human insulin. It involves gene synthesis, construction of expression plasmid and engineering cells ; isolation, purification and conversion of expressed products to form crystallized human insulin. Expression of porcine single chain insulin reached 40 - 50 mg / L with a final yield of 15 - 20 mg / L of crystallized human insulin after conversion. This expression and conversion rate could be further increased. This invention could be developed in the genetic engineering industry for human insulin production.

Claims

1. A method for the secretary expression of a single chain insulin precursor in yeast and its conversion to human insulin. It involved the synthesis of PSCI gene, yeast alpha mating factor leader sequence (α–MFL) ; cloning of PVT102-U / α–MFL- PSCI plasmid and the plasmid pPGK / PSCI ; inserton of this integrated plasmid into yeast cell to form engineering cell YS90 and YS91 ; the conditions for the separation and purification of the secretary expression product - PSCI , and its conversion to human insulin by transpeptidation. The specifics are as follows;

    a. T4 DNA ligase was used to link up chemically synthesized DNA fragments to a 175 base pair large DNA fragment (PSCI gene) of the following sequence;

L D K [redacted] R F V N Q H L C [redacted] S H L V
CTAGATAAAAGATTCGTTAACCAACACTTGTGCGGTTCCCACTT.GGTT
TATTTTCTAAGCAATTGGTTGTGAACACGCCAAGGGTGAA-CCAA

E A L Y L V C G E R G F F Y T P
G-AAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCT.TCTACACTC-CT-
C.TTCGAAACATGAACCAAACGCCACTTTCTCCAAAGA-AGATGTGAG.GA-

K A A K G I V E Q C C T S I C S
AAGGCTGCTAAGGGTATTGTCGAACAATGCTGTACC.TCCATCTG-CTCC-
TTCCGACGATTCCCATAACAGCTTGTTACGACATGG-AGGTAGAC.GAGG-

L Y Q L E N Y C N
TTGTACCAATTGGAAAACTACTGCAACTAGA
AACTAGGTTAACCTTTTGATGACGTTGATCTTCGA

This fragment contains codon for Leu, Asp, Arg and the gene of porcine single chain insulin precursor. There are XbaI and Hind III restriction enzyme sites at its 5' and 3' sticky ends.

PSCI were porcine B (1-30)-Xn-Y-porcine A (1-21), or porcine B (1-29) -Wn-Xn-Y-porcine A (1-21) ; W and X can be any natural amino acid excluding Lys and Arg ; Y is Lys or Arg ; n = 1 to 15

Known? Novo patent

b. The PSCI gene was cloned into PVT102-U / α - MFL to obtain the secretion expression plasmid PVT102-U / α - MFL - PSCI, with alcohol dehydrogenase I promoter (ADHp) and alcohol dehydrogenase I terminator sequence (ADHt).

c. Use phospoglycerate kinase promoter (PGKp) to replace ADHp in PVT102-U / α - MFL - PSCI to form the integrated plasmid pPKG / PSCI ;

d. DNA fragment of yeast α-mating factor (α - MFL) leader sequence were obtained by PCR with yeast DNA as template. PCR product obtained has the following α - MFL DNA sequence ;

CGATCCATGAGATTTCCTTCA.....AAAGAAGAAGGGGTATCTCTAGA
CCTAGGTACTCTAAAGGAAGT.....T TTCT TCT TCCCCATAGAGATCT

In comparison with the original α - MFL sequence , BamH I site was added in front of base No. 1 and leucine codon TTG (base No. 244-246) was changed to CTA to form an Xba I site.

e. Expression plasmid pVT102U /α - MFL-PSCI from b above was integrated into yeast cell *Saccharomyces cerevisiae* (XV-700-6B) to form the engineering cell *Saccharomyces cerevisiae* YS90, pVT /α - MFL-SCI (XV -700 -6B);

f. Expression plasmid pPKF / PSCI from c above was integrated into yeast cell *Saccharomyces cerevisiae* (XV-700-6B) to form the engineering cell *Saccharomyces cerevisiae* YS91, pPGK / SCI (XV -700 -6B);

g. Conversion and transpeptidation of secreted PSCI into insulin were carried out at 25°C for 6 hours in DMSO / 1,4 - butandiol / water.

Description :

Secretary expression of a single chain insulin precursor in yeast and its conversion into human insulin :

Insulin is a protein hormone with 2 polypeptide chains, A and B. In vivo, a single chain preproinsulin is formed before its conversion to the single chain proinsulin and subsequently, insulin (Steiner, D.F. et. al., Fed.Proc.Fed.Am.Soc.Exp.Biol., 1974, **33**: 2105 ; Science, 1967, **157** : 697)

Insulin is highly effective in treating diabetes, and it has been used clinically for over 70 years (Banting, F.G. & Best, C.H., J. Lab. Clin. Med., 1922, 7 : 251 ; Banting, F.G., et al., Canadian Med. Assoc. J., 1922 **12**: 141) . There is a great demand for insulin as there is a high occurrence of diabetes in the world population, and insulin is by far the largest volume peptide pharmaceutical used in clinical treatment. Therefore, any production and research related to insulin have always attracted great attention and interests. In the production of insulin , revolutionary and improved manufacturing methods as well as latest scientific discovery have been applied. Insulin was one of the first proteins to be expressed since the discovery of recombinant DNA technology (Ullrich, A. et al., Science, 1977, **196**: 1313 ; Crea, R. et al., Proc. Nat. Acad. Sci. USA, 1978, **75**: 5764). Genetically engineered insulin was the first biotechnological pharmaceutical to be released into the market place; and it has been gradually replacing the traditional animal insulins extracted from pancreas of domestic animals in the market (Klausner, A., Bio/Technology 1993, **11**: S35)

There have been many reports on genetic engineering of insulin, and for those which could be applicable in production, they could be grouped under 3 categories: Two of these categories concerned development with E-coli system , i.e. insulin is expressed as fusion protein in cytomplasmic inclusion bodies (Goeddel, D.V.; et al., Proc. Nat. Amer. Sci. USA, 1979, **76** : 106; Chance, R.E., et al., peptide: Synthesis-Structure-Ffunction, 1981 ed. D. H. Rich & E. Gross, pp. 721-728, Pierce Chemical Co., Rockford, IL; Chance R.E., et al., Diabetes Care, 1981, 4:147), or the expression product is connected to a signal peptide and secreted out of the cell (Talmadge, K., et al., Proc. Nat. Amer. Soci. USA, 1980, **77**: 3988; Chan S.J., et al., Proc. Nat. Amer. Soc. USA, 1981, **78**: 5401) . The third category was using the yeast system, where the expressed product was secreted into the medium through the cell wall (Markussen, J., et6 al., Diabetologia, 1986, 29: 568A; Thim L., et al., Proc. Nat. Amer. Sci. USA , 1986 , **83**: 6766 ;

Markussen, J., et al., Protein Engineering, 1987, 1:215). Exp i products were then modified and converted to insulin. Markussen's earlier work is under the same category of our invention here. His patent and related reports could be found in : Markussen, J., et al (1985), European Patent O 427 296 AL. Markussen, J., et al (1987), protein Engineering 1, (3)215. Markussen, J., et al (1986), peptides 1986, Walter de Gruyter & CO; Berlin - New York, pp189-194.

This invention used yeast to secret expression product of chemically synthesized porcine single chain insulin (PSCI) precursor gene , followed by in vitro transpeptidation of PSCI to human insulin. PSCI is porcine B (1-30)-Xn-Y-porcine A (1-21), or porcine B (1-29) -Wn-Xn-Y-porcine A (1-21) ; W and X can be any natural amino acid excluding Lys and Arg ; Y is Lys or Arg ; n = 1 to 15

Plasmid used for the transformation of yeast was constructed from pVT plasmid (Vernet, T., et al., gene, 1987, **52**: 225). PSCI was synthesized with a synthesizer. DNA fragment of yeast α-mating factor (α - MFL) leader sequence were obtained by PCR with yeast genomic DNA as template. After PSCI gene was ligated with α - MFL DNA fragment, it was inserted into the multi-cloning site between the alcohol dehydrogenase promoter (ADHp) and 3' terminating sequence of plasmid pVT102-U, to form the secretary expression plasmid pVT102U / α - MFL -PSCI. Phosphoglycerate kinase promoter (PGKp) was then used to replace ADHp in pVT102U / α - MFL -PSCI to obtain another secretary expression plasmid pPGK / PSCI . Yeast *Saccharomyces cerevisiae* (XV-700-6B) was used to form the engineering cells with the above plasmids: *Saccharomyces cerevisiae* YS90, pVT102U / α - MFL -PSCI (XV-700-6B) (CCTCC No. M93050)) and *Saccharomyces cerevisiae* YS91, pPGK / PSCI (XV-700-6B), (CCTCC No. M93051) (Stored in Chinese Traditional cell Culture Preservation Centre, Wuhan University, Wuhan). The secretary expression of porcine insulin precursor from transformed yeast cells reached 40 - 50 mg / L, after simple separation and purification. Separation, purification and transpeptidation of the expressed products yielded 15 - 20 mg / L of crystallized insulin. Its amino acid composition and sequence was the same as human insulin, and its receptor binding and biological activity were the same as native porcine insulin.

Description of the attached figures to this invention;

Figure 1: Agarose gel Electrophoresis of α - MFL DNA fragments after PCR

1. Hind III restriction enzyme fractions of ϕX 174
2. PCR Product
3,4 Purified PCR Product
5 Hae III restriction enzyme fragments of pBR322

Figure 2: pVT102U / α - MFL -PSCI transformed cells in situ hybridization

Figure 3: Construction of pVT102U / α - MFL -PSCI plasmid

Figure 4: 1.4% agarose gel electrophoresis

1. Hind III restriction enzyme fractions of ϕX 174
2. PCR of PGKp with yeast genome as template. Main band at 620 bp (fragment 1)

Figure 5: Restriction Enzyme fragments in 1% Agasrose gel electrophoresis

1. Hind III restriction enzyme fragments of λ-DNA
2. Eco R I + Sph I restriction enzyme fragments of pVT102U / α - MFL -PSCI.
3. Eco RI + BamH I restriction enzyme fragments of pVT102U / α - MFL -PSCI

Figure 6: Construction of pPGK / PSCI plasmid

Figure 7: Autoradiography result from Dot Blotting

a. Primer S as probe
b. PSCI fragment as probe.

Figure 8: 1% Agarose Gel electrophoresis of restriction enzyme fragments of pVT102U / α - MFL -PSCI and pPGK / PSCI.

1. MW markers ; pBR328 DNA with Bgl I + pBR322 DNA + Hinf I
2. Sph I restriction enzyme fragments of pVT102U / α - MFL -PSCI
3. BamH I fragments of pPGK / PSCI
4. Spn I + BamH I fragments of pVT102U / α - MFL -PSCI
5. Sph I + BamH I fragments of pPGK / PSCI
6. pVT102U / α - MFL -PSCI plasmid.

Figure 9 : PSCI : Sephadex G-50 (2.6 X 150 cm) gel filtration profile

Figure 10: PAGE of PSCI

1. PSCI
2. Porcine Insulin

Figure 11: PSCI Crystals (Ethanol System)

Figure 12: HPLC of 6 hours transpeptidation reaction mixture

Peak 1:18.29 minutes, PSCI
Peak 2: 19.95 minutes, DAI,
Peak 3:26.24 minutes, B30-butylated threonine , human insulin.

Figure 13: PAGE of transpeptidation reaction mixture:

a. Reaction mixture at time 0
b. Reaction mixture after 2 hours
c. Mixture of B30-butylated threonine human insulin and porcine insulin
d. Reaction mixture after 4 hours
e. Reaction mixture after 6 hours.

Figure 14: HPLC of B30-butylated threonine - human insulin after TFA treatment

Figure 15: PAGE of B30-butylated threonine - human insulin after TFA treatment

a. PSCI
b. Transpeptidation reaction products
c. B30-butylated threonine - human insulin
d. Human insulin
e. Mixture of B30-butylated threonine human insulin and Porcine insulin

Figure 16: Human Insulin Crystals.

Figure 17: Binding profiles between genetically engineered human insulin and porcine insulin with human placenta membrane insulin receptors.

The technical specification of this invention are detailed in the following application examples ;

Application Example I:
Construction of PSCI precursor gene secretary expression plasmid

1) Microbial Strains, plasmids and cloning procedure:

Cloning procedure according to standard method (Sambrook, J., et al., Molecular Cloning - A Laboratory Manual, 2nd ed. 1989, Cold Spring Harbor Laboratory Press, New York). E-coli JM103 was grown in 2YT medium and transformed by the calcium chloride procedure. Transformed E-coli cells were grown in 2YT medium containing ampicillin (100 ug / ml) Plasmid pVT102U was a gift from Thierry Vernet (Vernet, T., et al., Gene , 1987, **52:** 225) .Helper phage R408 was a gift from Michael Smith of University of Britishy Columbia .*Saccharomyces cerevisiae* XV700-6B (Leu-2, Ura-3, pep-4) , also kindly provided by Michael Smith, was grown in YPD medium and transformed by the lithium acetate method (Broker, M., Biotechniques, 1987, 5:516) . Transformed yeast cells were selected on a selecting medium containing 2% agar, 0.67% yeast nitrogen base, 2% glucose and amino acids (200 ug / ml each). Transformed yeast cells were cultured at 30°C , in YPD medium.

2) Construction of secretary expression plasmid pVT102U / α - MFL -PSCI .

2.1 Preparation of α - MFL DNA by PCR and its cloning in E-coli :

PCR (Mullis, K.B. & Faloona, F.A., Methods in Enzymology, 1987, 155:335) was performed manually in 3 water baths of different temperatures. 100 ul buffered reaction mixture contained 5 ul each of 10 mM dATP, dGTP, dCTP and dTTP, 1 ug template DNA and 10 - 50 pmol of each primer. The mixture was heated at 100°C for 5 minutes and annealed at 45°C for 2 minutes. 2.5 U taq DNA polymerase was added and the primers were elongated at 72°C for 1.5 minute. 25 to 35 PCR cycles were carried out. Each cycle consisted of 0.5 min. denaturation at 92°C . 1 min renaturation at 45°C and 1.5 min elongation at72°C. The size and yield of PCR product were examined by agarose gel electrophoresis with 5 ul reaction mixture.

Primer B (GGATCCATGAGATTTCCTTCA) and Primer X (TCTAGAGATA CCCTTCTTCTTT) were synthesized with ABI 380A DNA synthesizer and purified by gel electrophoresis (16% polyacrylamide-8 M urea). The modified α -MFL obtained had the following sequence :

CGATCCATGAGATTTCCTTCA.....AAAGAAGAAGGGGTATCTCTAGA
CCTAGGTACTCTAAAGGAAGT.....T TTCT TCT TCCCCATAGAGATCT

In comparison with the original α - MFL sequence , BamH I site was added in front of base No. 1 and leucine codon TTG (base No. 244-246) was changed to CTA to form an Xba I site. Agarose gel electrophoresis showed that the major product of PCR was α - MFL DNA fragment with about 250 bp (Figure 1). This was cloned into pVT102U to form pVT102U / α - MFL .

Shuttle plasmid pVT series (Vernet, T., et al., Gene, 1987, **52**: 225) has alcohol dehydrogenase I promoter (ADHp) and 3' terminating sequence, with multiple cloning sites (MCS) in-between. Single stranded DNA was obtained from pVT after superinfection with helper phage R408 (Russel, M. et al., Gene 1986 , **45**: 335), and it was used directly for DNA sequence analysis or point mutation. DNA sequencing was performed by Sanger's method (Sanger, F.G., et al., Proc. Nat. Amer. Sci. USA, 1977, **74**:5463) .

Amplification of α - MFL sequence, mutation and the addition of restriction enzyme sites at both ends were achieved at the same time by PCR.

2.2 Synthesis of PSCI gene and its cloning in E-coli

Eight fragments of oligonucleotides were synthesized with ABI 380A DNA synthesizer, purified by gel electrophoresis (16% polyacrylamide- 8 M urea) and ligated with T4 DNA ligase to form a large fragment of 175 bp of the following sequence;

L D K [redacted] F V N Q H L C [redacted] S H L V
CTAGATAAAAGATTCGTTAACCAACACTTGTGCGGTTCCCACTT.GGTT
TATTTTCTAAGCAATTGGTTGTGAACACGCCAAGGGTGAA-CCAA

E A L Y L V C G E R G F F Y T P
G-AAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCT.TCTACACTC-CT-
C.TTCGAAACATGAACCAAACGCCACTTTCTCCAAAGA-AGATGTGAG.GA-

K A A K G I V E Q C C T S I C S
AAGGCTGCTAAGGGTATTGTCGAACAATGCTGTACC.TCCATCTG-CTCC-
TTCCGACGATTCCCATAACAGCTTGTTACGACATGG-AGGTAGAC.GAGG-

L Y Q L E N Y C N
TTGTACCAATTGGAAAACTACTGCAACTAGA
AACTAGGTTAACCTTTTGATGACGTTGATCTTCGA

This fragment contains codon for Leu, Asp, Arg and the gene of porcine single chain insulin precursor. There are XbaI and Hind III restriction enzyme sites at its 5' and 3' sticky ends. It was inserted through T4 DNA ligase after the α–MFL of pVT102 U/ α - MFL to form of pVT102 U/ α - MFL -PSCI .The latter was used to transform E-coli JM 105. The transformants were selected by in situ colony hybridization with a probe of high specific radioactivity prepared by PCR using PSCI gene as a template to incorporate ^{32}P (Figure 2).

Single stranded DNA of of pVT102 U/ α - MFL-PSCI was prepared (Russel, M. et al., Gene 1986 , **45**: 335) and the sequence of PSCI gene was confirmed by DNA sequencing.

Figure 3 shows the construction of pVT102 U/ α - MFL -PSCI from pVT102-U.

3. Construction of secretary expression plasmid pPGK / PSCI

Phospoglycerate kinase gene (PGK) is one of the most efficiently expressed gene in yeast. PGK promoter (PKGp) has been used to construct a series of high-efficiency expression carriers. To replace the ADHp in pVT102 U/ α - MFL-PSCI with PGKp, PGKp sequence was first amplified (Ogden, J.E., et al., Mol. Cell. Biol., 1986, 6:4335) in the yeast genome by PCR (615 bp), and Sph I and BamH I restriction sites were added to its 5' and 3' ends respectively (Figure 4). Primer S (CCGCATGCTCA TACTATT-ATCAGGGCCA) and primer B (GGGGATCCTGTTTTTATATTTGTT GTAAAA) were synthesized with ABI 380A DNA synthesizer, and purified by gel electrophoresis (16% polyacrylamide- 8M urea).

3 fragments of 1.2 Kb, 1.85Kb and 4.25Kb were obtained when pVT102 U/ α - MFL -PSCI was treated with EcoR I and Sph I. 1.85 Kb fragment was recovered (Figure 5). Two fragments of 2.5 Kb and 5 Kb were obtained when treated with EcoR I and BamH I, and 5Kb fragment was recovered in this case (Figure 5).

The recovered 5 Kb and 1.85 Kb fragments were mixed and ligated with the PGKp fragment from PCR to obtain the secretary expression plasmid pPKG / PSCI . Figure 6 shows the construction of this plasmid.

E-coli JM103 was transformed with pPKG / PSCI. DNA plasmids were extracted from 20 transformed colonies and dotted on nylon membrane . Dot hybridization were carried out with primer S and PSCI fragments from PGKp amplification step (Figure 7). Restriction enzyme map of transformed plasmid DNA is shown in figure 8. Promoters from pPKG / PSCI and pVT102 U/ α - MFL -PSCI could be removed with restriction enzymes Sph I + BamH I. Their sizes are 600bp and 400bp respectively, and they are similar in size to PGKp and ADHp (PGKp is 620 bp and ADHp is 415 bp ((Figure 8).

Application Example 2
Secretary Expression of PSCI in yeast cell

Yeast cells *Saccharomyces cerevisiae* XV700-6B transformed by pPGK / PSCI or pVT102 U/ α - MFL -PSCI (YS-91 or YS-90) were grown for 48 hours at 30°C in selective medium. Transformed cells were transferred into 5ml YPD medium and grown overnight. This was then transferred into 50ml YPD medium for 20 hours, followed by growing in roller bottles or 10L fermenter for 3 days in medium with YPD. The concentration of PSCI in roller bottles and the 10L fermenter with YS91 were 24 mg / L and 40 - 50 mg / L respectively, when determined by insulin radioimmunoassay.

Application Example 3
Isolation, Purification and Determination of the expressed PSCI

PSCI were precipitated from the culture medium by acetone or trichloroacetic acid and kept at 4°C overnight. Precipitate was collected after centrifugation and dissolved in small amount of acetic acid before purified by gel filtration through Sephadex G-50 column with 10% acetic acid. Fractions eluted corresponding to insulin were collected (Figure 9), and lyophilized. This gel filtration and lyophilization were repeated once more and the product obtained showed one single band with PAGE (Figure 10) . Crystallized PSCI (ethanol system, pH 6.8) as shown in figure 11 was obtained from this product.

30 - 40 mg of pure PSCI could be obtained from 1 L of fermentation broth with the above purification procedure.

Application Example 4
Transpeptidation and Characterization of Human Insulin

4.1 Transpeptidation

PSCI was dissolved in DMSO / 1,4-butanediol. Thr(Bu^t)-OBu^t was added in excess and the pH was adjusted to 6.5 . TPCK-trypsin was added (substrate:enzyme = 5:1) and the reaction mixture was incubated at 25°C for 6 hr. The reaction was stopped by acidification (Zhu, S.Q. and Cui, D.F., Ke Xue Tong Bao, 1984, **20**: 1263). Mixture showed 3 major peaks after HPLC (Figure 12); Peak 1 was unreacted PSCI, peak 2 was des-B30 insulin (DAI, des-B30 ala) and peak 3 was the transpeptidated product, [B30Thr(Bu^t)-OBu^t] human insulin. Figure 13 is PAGE of the reaction mixture.

According to calculation from the peaks in figure 12, [B30Thr(Bu^t)-OBu^t] human insulin was 51%, PSCI was 41% and DAI was 8%. PSCI and DAI could be recovered for transpeptidation.

[B30Thr(Bu^t)-OBu^t] human insulin was deblocked with TFA to obtain human insulin. Human insulin obtained showed a single peak after HPLC (Figure 14), and the recovery was closed to 100%. Figure 15 showed PAGE result of [B30-Thr(Bu^t)-OBu^t] human insulin after TFA.

15 -20 mg of pure human insulin was obtained under the above transpeptidation conditions. However, the 41% PSCI and 8% DAI (Figure 12) could be recovered for transpeptidation. Therefore, the final yield of human insulin could be further increased.

4.2 Characterization of the genetic engineered human insulin:

Crystalized human insulin obtained after transpeptidation is shown in figure 16. Experimental values obtained from amino acid composition analysis correspond well with the theoretical values of insulins (table 1):

Genetically engineered human insulin amino acid sequence was determined by ABI protein sequencer and found to be the same as native human insulin .

The binding activity of genetically engineered human insulin and porcine insulin with insulin receptors from human placental membrane were found to be identical (Feng, Y.M., et al Biochem. Biophy. Acta Sinica, 1982, **14**: 137). Their in vivo biological activity when determined by a semi-quantitative ICR mice convulsion assay were also found to be identical. (Table 2).

Table 1 : Amino acid composition analysis of Porcine insulin (P-Ins) and human insulin (H-Ins) :

Amino Acids	Porcine Insulin		Human Insulin	
	Exp. Value	Theo. Value	Exp. Value	Theo. Value
Asx	3.08	3	3.21	3
Thr	1.99	2	3.10	3
Ser	2.59	3	2.66	3
Glx	7.00	7	7.63	7
Gly	4.00	4	4.00	4
Ala	2.12	2	1.31	1
Val	3.69	4	4.00	4
Ile	1.70	2	1.80	2
Leu	5.98	6	6.03	6
Tyr	3.81	4	3.58	4
Phe	2.86	3	3.14	3
His	1.86	2	2.00	2
Lys	1.02	1	1.16	1
Arg	1.06	1	1.10	1
Pro	0.64	1	0.50	1

Note: Samples were hydrolyzed in 6M HCl for 20 hours.

Table 2: Semi-quantitative mouse convulsion assay between porcine insulin (MC-Ins) and genetic engineered human insulin (h-Ins)

Sample (ug)	h-Ins	MC-Ins
0.5	6 / 10	6 / 10
0.25	1 / 10	1 / 10

Porcine and human insulin only differed in one single amino acid at B30 position; where it is Ala for porcine insulin and threonine for human insulin. Therefore, porcine insulin can be obtained after transpeptidation in the presence of butylated alanine ($AlaOBu^t$) instead of butylated threonine ($ThrOBu^t$).

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